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Aminoalkylindole Binding in Rat Cerebellum: Selective Displacement by Natural and Synthetic Cannabinoids

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ABSTRACT

A binding assay for WIN 55212-2, an aminoalkylindole (AAI) with antinociceptive activity in rodents, is described. [^{3}H]WIN 55212-2 bound to rat cerebellar membranes with a K_d of 2 nM and a maximum binding of 1.2 pmol/mg of protein. Specific binding in this filtration assay was greater than 90%, saturable, reversible, stereospecific, pH sensitive and heat labile. Binding was decreased by Na^+, K^+, Li^+ and nonhydrolyzable analogs of GTP and increased by Mg^{++} and Ca^{++}. The density of specific binding sites varied throughout the central nervous system with the highest found in the cerebellum, hippocampus and striatum and the lowest in the medulla/pons and spinal cord. The binding affinities of other AAIs for the WIN 55212-2 binding site correlated with their potencies for inhibiting neuronally stimulated contractions in the isolated mouse vas deferens. Of more than 60 compounds representing recognized neurotransmitter systems, only cannabinoids effectively inhibited binding. The effect of cannabinoids on AAI binding was consistent with competitive inhibition and suggests that AAI activity may be mediated in whole or in part by interaction with cannabinoid receptors. AAIs appear to represent a structurally novel class of compounds with which to study cannabinoid receptors.

AAIs were designed, from the prototype pravadoline, as potential analgesics which would lack the gastrointestinal side effects associated with NSAIDs. AAIs are similar in structure to some NSAIDs, *e.g.*, indomethacin, but differ by having an aminoalkyl group in place of the acidic functional group and typically an aroyl group at the indole 3-position (Bell *et al.*, 1991). In several animal models involving acute chemical, thermal or pressure nociceptive stimuli, pravadoline exhibited more diverse and greater antinociceptive efficacy than NSAIDs (Haubrich *et al.*, 1990). It also possesses analgesic efficacy against postoperative pain in humans (Grieco *et al.*, 1989).

Although pravadoline inhibits prostaglandin formation, this property does not appear to account completely for its analgesic profile (Haubrich *et al.*, 1990); other AAIs demonstrate greater antinociceptive activity in rodents without inhibiting mouse brain cyclooxygenase *in vitro* (Ward *et al.*, 1988). Furthermore, it is unlikely that AAIs possess an opioid-based mechanism of antinociception (Haubrich *et al.*, 1990; Ward *et al.*, 1990b).

The *in vivo* antinociceptive potency of AAIs is correlated with their ability to inhibit neuronally stimulated contractions in the MVD preparation (Ward *et al.*, 1988, 1990b). The potency and structural selectivity demonstrated by various AAIs for inhibiting neuronally stimulated contractions in this preparation, a presynaptic effect, suggests a receptor-mediated mechanism of action (Ward *et al.*, 1988; Pacheco *et al.*, 1991). A receptor site of action is also suggested by the ability of compounds in this series to inhibit adenyl cyclase activity in rat cerebellar homogenates. Adenyl cyclase inhibition, observed under both basal and forskolin-stimulated conditions, is attenuated by pertussis toxin, dependent on magnesium and GTP, and not dependent on nonhydrolyzable GTP analogs (Pacheco *et al.*, 1991). Although this suggests that AAIs act through specific G-protein linked receptors, no significant interactions were observed in bioassays or radioligand binding assays involving purine, adrenergic, opioid, dopamine, τ-aminobutyric acid, serotonin, neurokinin, bradykinin, prostanoid or cholinergic receptors (Ward *et al.*, 1990b).

The purpose of the present study was to examine the binding properties of a radiolabeled, prototypic noncyclooxygenase inhibiting AAI, [^{3}H]WIN 55212-2, and to compare its binding properties with those of previously described neurotransmitter receptor systems.

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ABBREVIATIONS: AAI, aminoalkylindole; NSAIDs, nonsteroidal anti-inflammatory drugs; MVD, mouse vas deferens; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMSO, dimethylsulfoxide; BSA, bovine serum albumin; B_{max}, maximum binding; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; GTP-γ-S, guanosine 5'-O-(3-thiophosphate); ATP-γ-S, adenosine 5'-O-(3-thiophosphate); GppNHp, guanosine 5'-(β,γ-imido)triphosphate; AppNHp, adenosine 5'-(β,γ-imido)triphosphate; DALN, desacetyllevonantradol; 5-HT, 5-hydroxytryptamine; THC, tetrahydrocannabinol.

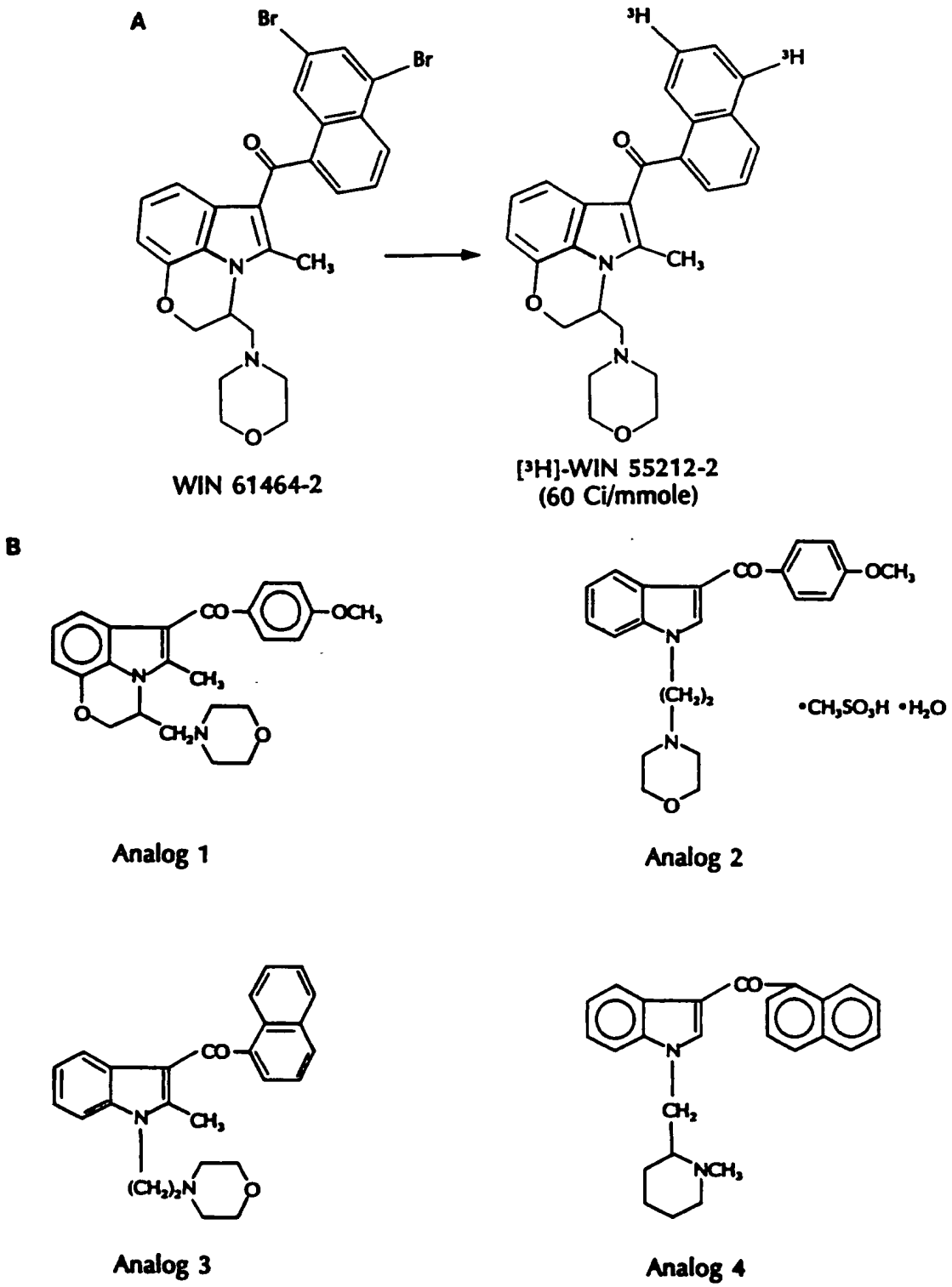

Fig. 1. A, structure of AAI radioligand. (*R*)-(+)-[^{3}H]WIN 55212-2 was synthesized (under contract by DuPont-New England Nuclear) from its dibrominated precursor, WIN 61,464-2, by catalytic exchange. The specific activity of the radiolabeled product was 59–60 Ci/mmol. B, structures of AAI analogs.

Methods

AAI binding assay. Male Sprague-Dawley rats (Taconic Farms, Inc., Germantown, NY), weighing 200–250 g, were decapitated and their brains excised rapidly. The meninges were removed and brain regions dissected on ice. Tissue was stored for up to 1 month at −70°C with no apparent change in binding. Tissue was homogenized (Brinkmann Polytron; Westbury, NY) in ice-cold 20 mM HEPES-NaOH, pH

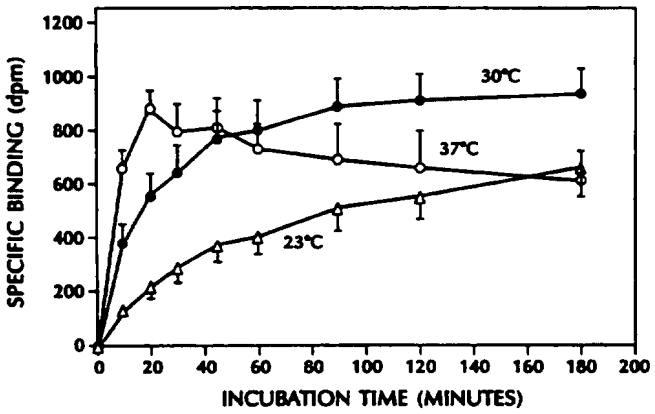

Fig. 2. [^{3}H]WIN 55212-2 binding equilibrium. The effect of temperature on time required to reach equilibrium and on the stability of specific binding of 0.1 nM [^{3}H]WIN 55212-2 in rat cerebellar homogenate was determined in the presence of 100 μg of protein. Data points are the means ± S.E. of three separate experiments, each performed in triplicate.

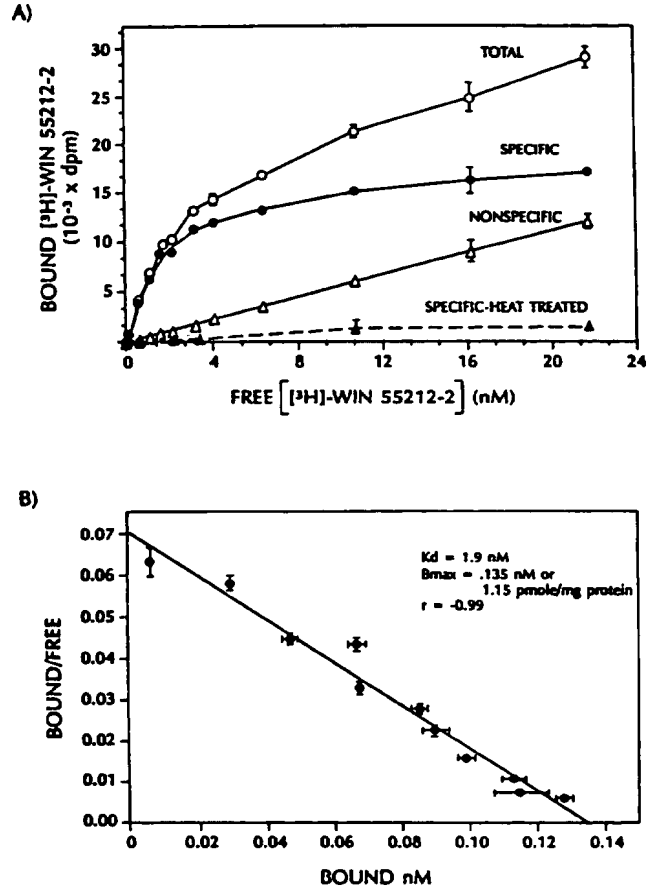

Fig. 3. Saturable specific binding of [^{3}H]WIN 55212-2 in rat cerebellar homogenate. A, nonspecific binding was determined by using 1 μM WIN 55212-2. Heat treatment of homogenate (55–60°C for 25 min) reduced specific binding to 9.0 ± 2.2% of control. Data points are the means ± S.E. of three separate experiments, each performed in triplicate. B, Scatchard analysis of specific binding shown in A. Free radioligand concentration was calculated indirectly from total concentration minus specific bound; however, when free radioligand concentration was measured directly and used in the Scatchard transformation, no change in K_d or B_{max} was found. Parameters and best fit line were determined by least-squares linear regression analysis.

7.0, diluted approximately 1:100 (w/v) with the same buffer and centrifuged at 48,000 × *g* for 10 min at 4°C. The pellet was washed by resuspension and centrifugation as above. The final pellet was suspended (1:120 w/v) in 20 mM HEPES-NaOH, pH 7.0, and stored on ice for use within 1 hr.

AAIs (10 mM) were routinely solubilized in a mixture of 0.3 N methanesulfonic acid-13.5% ethanol. AAIs (10 mM) were insoluble in water, in 0.5 N HCl, in 0.5 N lactic acid and in 0.35 N methanesulfonic acid. Cannabinoids were dissolved in absolute ethanol. Other compounds were dissolved in DMSO. Dissolving WIN 55212-2 in the same vehicle as the cannabinoids had no significant effect on its rank order relative to cannabinoids in competition experiments. All compounds, including [^{3}H]WIN 55212-2 (stored in absolute ethanol), were diluted in 20 mM HEPES, pH 7.0, containing 5 mg/ml of BSA. BSA prevented adsorption of AAIs and cannabinoids to glassware. The use of Prosil-treated glass, polystyrene or polypropylene tubes was unnecessary in the presence of BSA and inadequate in its absence.

The final assay concentration of BSA was 1.0 mg/ml in these experiments; however, recent studies indicate that a final concentration of 0.2 mg/ml is as effective in preventing adsorption and may result in higher specific binding. Final assay concentrations of ≤ 50 mM ethanol, ≤ 1×10^{-3} N methanesulfonic acid, ≤ 2.5 mM ethanol + 3×10^{-4} N methanesulfonic acid or ≤ 3% DMSO had no effect on specific binding. The possible effects of all vehicles, however, including BSA, were controlled in each assay.

The assay was started with the addition of homogenate and the tubes were incubated at 30°C for 90 min in a shaking waterbath unless otherwise noted. Each tube contained 20 mM HEPES-NaOH buffer, pH 7.0, 1.0 mg/ml of BSA, competing drug or vehicle and 100 to 125 μg of membrane protein in a 1-ml incubation volume. Saturation studies were conducted over 3 log U of radioligand concentration (0.1–22 nM), and competition studies were performed in the presence of 0.5 nM [^{3}H] WIN 55212-2. Specific binding was defined as the difference in binding in the presence and absence of 1.0 μM unlabeled WIN 55212-2.

Samples were filtered rapidly over Whatman GF/B filters (presoaked in 5 mg/ml of BSA-buffer) by using a 48-channel cell harvester (Brandel, Gaithersburg, MD). Filters were rinsed with 20 ml of 20 mM HEPES, pH 7.0, containing 0.5 mg/ml of BSA. Radioactivity on the filters, expressed in disintegrations per minute, was measured by liquid scintillation spectrometry (Beckman LS 5000TA) by using Biofluor (E. I. Dupont de Nemours and Co., Wilmington, DE) scintillation cocktail. Assays were conducted in triplicate and experiments were repeated at least 3 times. Protein was determined by the method of Lowry *et al.* (1951) (controlled for the effect of HEPES buffer) using BSA as a standard.

In order to filter samples simultaneously for dissociation kinetic studies using the cell harvester, 1 μM WIN 55212-2 was added at various time points to samples in which the radioligand binding had reached equilibrium. After equilibrium was reached, there was no difference in specific binding over the 2-hr time period in which dissociation was initiated. For association kinetic studies, homogenate was added at different time points and samples filtered simultaneously as described above. The concentration of radioligand in dissociation studies was 0.5 to 2 nM and in association studies was 0.5 to 5 nM. Less than 5% of the total radioligand was bound under these conditions (125 μg of protein).

Data analysis. All saturation and competition studies were analyzed by computer by using LIGAND (Munson and Rodbard, 1980), as modified with equilibrium binding data analysis (McPherson, 1983). Equilibrium dissociation constants (K_d and K_i) and B_{max} values for all AAIs and cannabinoids were obtained by one-site analysis in LIGAND. Slope factors (Hill coefficients) were obtained from nonlinear curve fitting to the four parameter logistic equation:

$$\text{Bound (dpm)} = \frac{(B_{max} - BG)}{(1 + [\text{Disp. Conc.}/\text{IC}_{50}]^P} + BG$$

where B_{max} is the total bound, BG is nonspecific bound, P is the slope

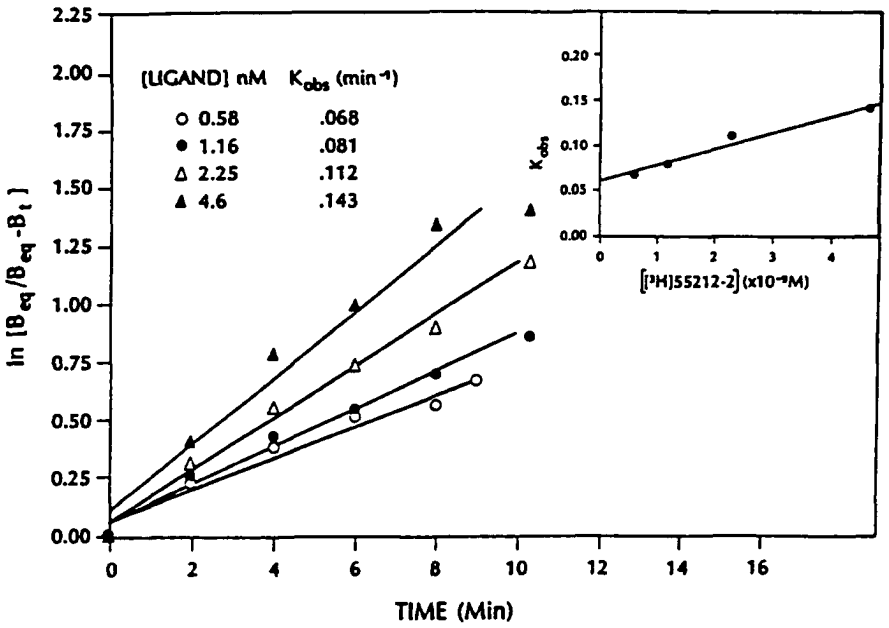

Fig. 4. [³H]WIN 55212-2 association kinetics in rat cerebellar homogenate (100 μg of protein). Radioligand concentration was varied between 0.58 to 4.6 nM and nonspecific binding was determined at each time point by using 1 μM WIN 55212-2. K_{obs} was determined as described by Bennett and Yamamura (1985). Ln[B_{eq}/(B_{eq} − B_t)], where B_{eq} is specifically bound radioligand at equilibrium and B_t is the specifically bound radioligand at time t, is plotted *vs.* time yielding a line whose slope is K_{obs}. The inset plot of K_{obs} *vs.* radioligand concentration yields a line whose slope is the association rate constant and y-intercept is the dissociation rate constant. In the representative experiment (of three independent studies) shown K_{on} = 1.86 × 10⁷ min⁻¹ M⁻¹; K_{off} = 0.061 min⁻¹; K_d = 3.3 nM and r = 0.98.

factor (Hill coefficient) and IC_{50} is the concentration of displacer (Disp. Conc.) inhibiting 50% of specific binding (McPherson, 1985; see also Barlow and Blake, 1989).

Association kinetic studies were analyzed graphically using semi-ln plots to determine k_{obs} values at each radioligand concentration; the k_{on} value was then determined indirectly by replotting k_{obs} *vs.* radioligand concentration (Bennett and Yamamura, 1985). Dissociation data were analyzed by the computer program KINETIC (McPherson, 1983).

MVD bioassay. Vasa deferentia preparations from Swiss-Webster mice were incubated at 37°C, in oxygenated (95% O_2-5% CO_2) modified (no Mg^{++}) Krebs' solution. Preparations were mounted at a resting tension of 500 mg and stimulated electrically at the minimum voltage required to produce a maximal effect (0.1 Hz twin pulses of 1 msec duration and 10 msec delay). Contractions were recorded isometrically (Grass FT 03 C transducer; Quincy, MA) on a polygraph recorder (Grass RPS 7c 8). After equilibration (30–60 min), solutions of compounds were added to the fluid bathing the tissue in 5- to 100-μl volumes. Agonist concentration-effect curves were generated cumulatively. The preincubation time with antagonist was 10 min. The washout time between agonist concentration-effect curves was a minimum of 40 min. IC_{50} values were determined by log-probit analysis of the data as described by Tallarida and Murray (1987).

Materials. (*R*)-(+)-[³H]WIN 55212-2 was prepared under contract by DuPont-New England Nuclear (Boston, MA) from the [³H]WIN 55212-2 precursor WIN 61464-2 (Sterling Winthrop Pharmaceuticals Research Division, Rensselaer, NY) by using a catalytic exchange reaction (fig. 1) (D'Ambra *et al.*, 1992). (*R*)-(+)-WIN 55212-2 was chosen for labeling because 1) it does not inhibit cyclooxygenase, 2) it does not inhibit binding in a variety of tested radioligand binding assays, 3) it is highly potent in isolated tissue and animal nociceptive studies and 4) its enantiomer possesses little biological activity (Ward *et al.*, 1988, 1990a; S. Ward and D. Luttinger, personal communication). Specific activity was 59–60 Ci/mmol and purity, determined by TLC and HPLC, was ≈99%. The radioligand was stable at 5.0 mCi/ml in ethanol at −80°C for periods ranging from 4 months to over 1 year. Purity was monitored at New England Nuclear by TLC before each shipment [solvent system-hexane-ethyl acetate (1:1) on silica gel] and by HPLC [25-cm Zorbax ODS column with a acetonitrile-H_2O (65:35) mobile phase]. Purity was also monitored in our laboratory using HPLC with a Waters resolve C_{18}, 10-μm column with 0.05 M ammonium acetate, 25 to 100% acetonitrile gradient. The eluates were identified by means of a UV detector at a wavelength of 315 nm and by liquid scintillation spectrometry.

GTP-γ-*S*, ATP-γ-*S*, GppNHp and AppNHp, each a tetralithium salt, were purchased from Boehringer Mannheim Corp. (New York, NY). Rimcazole was obtained from Burroughs-Wellcome Company (Research Triangle Park, NC), phencyclidine from National Institute on Drug Abuse (Rockville, MD), diltiazem from Marion Laboratories (Kansas City, MO), adenosine from Boehringer Mannheim, verapamil from Knoll Laboratories (Orange, NJ), ketanserin and haloperidol from Janssen Pharmaceutica (Beerse, Belgium), morphine from New York Quinine and Chemical Works, Inc. (New York, NY), taurine from Mann Fine Chemicals (New York, NY), pyrilamine from Merck Sharp and Dohme (West Point, PA), naloxone from Endo Laboratories, Inc. (Garden City, NY) , trazodone from Mead Johnson and Company (Evansville, IN), diazepam and RO 151788 from Hoffman-La Roche, Inc. (Nutley, NJ), baclofen from Ciba-Geigy Corp. (Summit, NJ), pindolol and bremazocine from Sandoz Pharmaceuticals (Hanover, NJ), propranolol from Ayerst Laboratories (New York, NY), L-glutamic acid and 3-3-(3-hydroxyphenyl)-N-propylpiperidine from Research Biochemicals, Inc. (Wayland, MA), 1,3-di(2-tolyl)guanidine from Aldrich Chemical Company, Inc. (Milwaukee, WI), tiotidine from Imperial Chemical Industries (Wilmslow, Cheshire, England), somatostatin from Peninsula Laboratories (San Carlos, CA), apomorphine from Merck & Company (Rahway, NJ) and SKF-10047 from Smith Kline and French Laboratories (Philadelphia, PA). Prazosin, DALN, CP55940 and CP56667 were provided by Pfizer, Inc (New York, NY). AAIs were obtained from Sterling Winthrop Pharmaceuticals Research Division. All other chemicals, including fatty-acid deficient BSA (A-7030), were obtained from Sigma Chemical Company (St. Louis, MO).

Results

Preliminary studies to determine appropriate conditions for AAI binding in rat cerebellar homogenate. The degree of specific binding of 0.5 nM [³H]WIN 55212-2 was similar (≥90% of total binding) in the following buffering systems (millmolar): HEPES-NaOH, 20, pH 7.3; Tris-HCl, 20, pH 7.3; or Tris-HCl 20, pH 7.3; containing NaCl, 120; KCl, 5; $CaCl_2$, 2; and $MgCl_2$, 1. Optimal binding in the HEPES buffer reached a plateau between pH 6.8 and 7.1 (data not shown). To permit further characterization of the effects of individual ions,

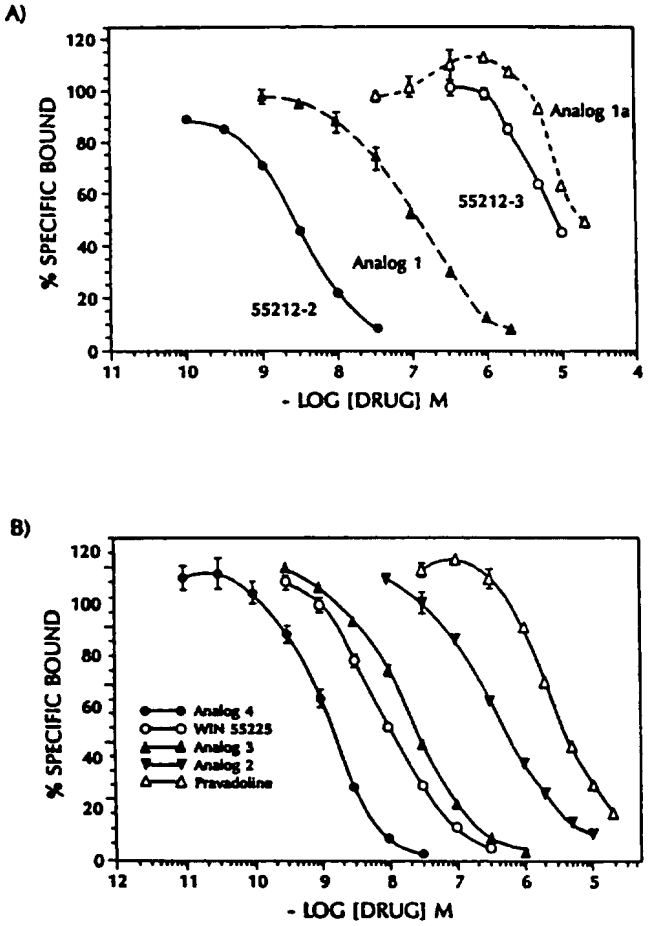

Fig. 5. Competition curves of AAIs for [^{3}H]WIN 55212-2 binding. A, competition curves of enantiomers of WIN 55212 and Analog 1 with [^{3}H] WIN 55212-2 (0.5 nM) showing stereospecific binding in rat cerebellar homogenate (100–120 μg of protein). Complete competition curves for WIN 55212-3 and Analog 1a could not be obtained because of vehicle effects at drug concentrations above 10 μM. B, competition curves of various aminoalkylindole agonists, with K_i values extending over > 3 log u were performed under the same conditions as in A. In both figures, each data point is mean ± S.E. of three independent experiments each performed in triplicate. See figure 9A for the structure of WIN 55225 and figure 1B for the structures of the other AAI analogs.

TABLE 1

AAI analogs: binding parameters in rat cerebellar membranes and IC_{50} values for inhibiting neuronally stimulated contractions in isolated MVD

See figures 1B and 9 for chemical structures. Each value is mean ± S.E. of at least three independent experiments.

AAI	Binding in Rat Cerebellum K_i	Slope Factor	MVD Assay IC_{50}
	nM		*nM*
Analog 4	0.76 ± .047	1.16 ± 0.045	0.22 ± 0.07
WIN 55212-2	2.2 ± 0.26	0.94 ± 0.051	0.40 ± 0.1
WIN 55225	7.4 ± 0.24	0.89 ± 0.009	6 ± 1.2
Analog 3	16 ± 1.21	0.84 ± 0.015	15 ± 1.7
Analog 1	97 ± 7.03	0.87 ± 0.034	44 ± 13
Analog 2	315 ± 20	0.83 ± 0.023	100 ± 6
Pravadoline	2511 ± 177	1.13 ± 0.064	319 ± 63

a 20 mM HEPES buffer, pH 7.0, was chosen for the studies described in this report.

Specific binding of 0.5 nM [^{3}H]WIN 55212-2 was linear over a protein range of 25 to 400 μg/ml; the linearity of specific binding using 20 nM radioligand extended to 900 μg/ml (data not shown). Experiments described here routinely used 100 to 125 μg of protein from the washed rat cerebellar homogenate.

The effects of temperature on the time required to reach equilibrium and on the stability of specific binding were determined by using 0.1 nM [^{3}H]WIN 55212-2 (fig. 2). At 30°C, equilibrium was reached by 90 min and was maintained for 3 hr. Although the same level of specific binding was reached after 20 min at 37°C, a gradual decrease of 30% in specific binding occurred by 3 hr. Binding at 23°C did not reach equilibrium by 3 hr and binding at 4°C was negligible (not shown).

Saturability of AAI binding. Specific binding of [^{3}H]WIN 55212-2 (0.1–22 nM) in rat cerebellar homogenate was saturable, whereas nonspecific binding, defined by using 1 μM unlabeled WIN 55212-2, increased linearly over the range of radioligand concentrations studied (fig. 3A). When a protein concentration of 100 μg/ml was used, nonspecific binding accounted for 6 to 8% of the total binding in the presence of 0.5 nM radioligand and 35 to 40% of total binding in the presence of 22 nM radiolabeled AAI. GF/B filters (presoaked in 5 mg/ml of BSA) contributed 40 to 50% of this nonspecific binding as determined in the absence of tissue homogenate. Heat treatment of the homogenate, 55–60°C for 25 min, reduced specific binding at saturating radioligand concentration (22 nM) to 9.0 ± 2.2% of the unheated preparation. This, as well as the pH sensitivity, is consistent with a protein binding site.

By using the iterative nonlinear least-squares curve fitting program LIGAND, the best fit for the saturable specific binding was a one-site model with K_d = 1.89 ± 0.091 nM and B_{max} = 1.15 ± 0.068 pmol/mg of protein (mean ± S.E. from regressions of each of three individual experiments, fig. 3A). These values are similar to those obtained from Scatchard transformation of the same data (K_d = 1.92 nM and B_{max} = 1.13 pmol/mg of protein), by using a least-squares linear regression analysis (fig. 3B). The receptor concentration used in saturation studies yielded a B_{max} value of 0.134 ± 0.0042 nM, a concentration more than 10-fold lower than the K_d for the radioligand. Furthermore, less than 6% of initial radioligand was bound.

Saturation experiments performed in the presence of 0.5 nM [^{3}H]WIN 55212-2 and increasing concentrations of unlabeled WIN 55212-2 (fig. 5A) yielded binding parameters in close agreement with radioligand saturation experiments: K_d = 2.2 ± 0.258 nM, B_{max} = 1.24 ± 0.091 pmol/mg of protein. With nonspecific binding determined as a parameter in the LIGAND analysis, a one-site model was the best fit.

A Hill transformation of radioligand saturation data indicated that [^{3}H]WIN 55212-2 labeled a single class of binding sites (n_H = 1.00 ± 0.032, n = 3); this is in agreement with the one-site fit obtained in LIGAND analysis. The Hill coefficient for cold saturation experiments was 0.959 ± 0.114 (n = 3). This apparent homogeneity would be expected when the unlabeled form of the radioligand is used to define nonspecific binding.

Kinetic analysis of AAI binding. Association studies were performed at radioligand concentrations in the range of 0.5 to 5.0 nM. Values for k_{obs} were determined from the slope at each concentration (fig. 4). An association rate constant of 1.33 ± 0.268 × 10^7 min^{-1} M^{-1} (three experiments) was obtained from the slope of the line on a graph of k_{obs} *vs.* ligand concentration (fig. 4, inset).

Dissociation studies, analyzed using the KINETIC computer program (MacPherson, 1983), indicated that [^{3}H]WIN 55212-

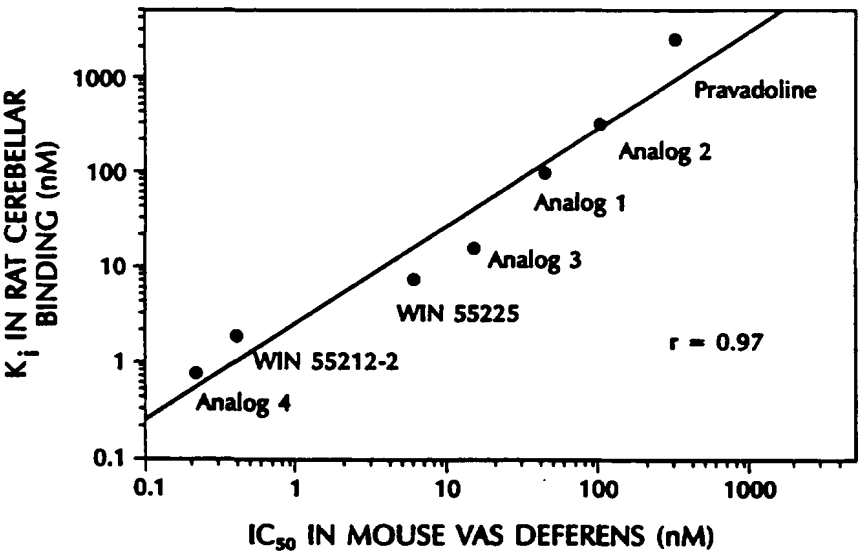

Fig. 6. Rank order potency of AAI agonists: Competition for [^{3}H]WIN 55212–2 binding (K_i) in rat cerebellar membranes *vs. in vitro* inhibition of neuronally stimulated contractions in MVD (IC_{50}). Least-squares linear regression was used to determine the best fit line. See figure 9A for the structure of WIN 55225 and figure 1B for the structures of the AAI analogs. Table 1 lists the IC_{50} or K_i values. See "Methods" and "Results" for details of procedure and analysis.

2 binds reversibly to the receptor in a statistically significant ($P < .01$) biphasic manner. The faster dissociation rate constant was 0.135 ± 0.028 min^{-1} ($n = 6$, $T_{1/2} = 5$ min); the slower rate constant was 0.018 ± 0.0018 min^{-1} ($n = 6$, $T_{1/2} = 39$ min) (data not shown). Because dissociation was initiated by addition of excess unlabeled ligand rather than infinite dilution, negative cooperativity can be ruled out as a possible explanation of the biphasic kinetics. The presence of independent binding sites or interconverting affinity states dependent on G-protein interactions awaits further investigation.

By using the association rate constant of 1.33×10^7 min^{-1} M^{-1} and the two dissociation rate constants described above, K_d values of 1.34 and 10.1 nM were calculated. The lower K_d is consistent with the value (1.89–2.2 nM) obtained from equilibrium binding studies.

Stereoselectivity of the [^{3}H]WIN 55212–2 binding site. Competition studies using enantiomers of two different AAIs indicate the stereospecific nature of AAI binding (fig. 5A). WIN 55212-2, the (*R*)-enantiomer, bound with more than 2500-fold greater affinity than its (*S*)-enantiomer, WIN 55212-3 (K_i = 6,300 ± 210 nM). The (*R*)-enantiomer of the 3-parametoxy benzoyl analog (Analog 1) of WIN 55212-2 bound with 140-fold greater affinity than its (*S*)-enantiomer (Analog 1A) (K_i = 13,857 ± 549 nM). Complete competition by WIN 55212-3 and Analog 1A for [^{3}H]WIN 55212-2 binding sites could not be achieved due to effects of the vehicles needed to solubilize these AAIs at or above 10 μM.

Competition for [^{3}H]WIN 55212–2 binding by AAI agonists. The ability of other AAIs to compete for the [^{3}H] WIN 55212-2 binding site in rat cerebellar homogenate is shown in figure 5B. These competition curves are monophasic and cover a 2500-fold range in binding affinities. Slope factors (Hill coefficients), obtained from a logistic equation (McPherson, 1985), all approached unity (table 1). As determined by using LIGAND analysis, a two-site model was not a significantly better fit ($P > .05$) than a one-site model for any of the competition curves.

The K_i values obtained from these competition studies and the IC_{50} values obtained from experiments measuring the inhibition of neuronally stimulated contractions of isolated MVD

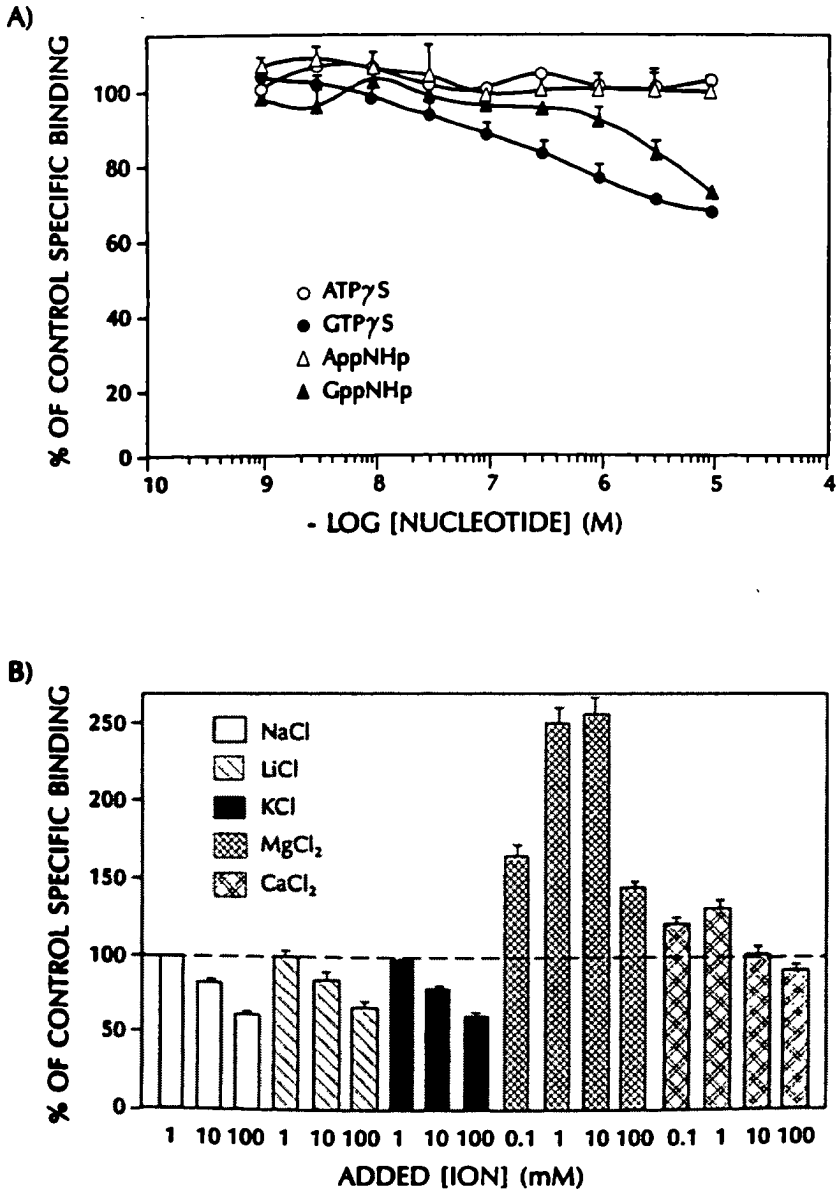

Fig. 7. A, effects of guanine and adenine nucleotides on the specific binding of [^{3}H]WIN 55212-2 (0.5 nM) in rat cerebellar homogenate. Each point is the mean ± S.E. of three independent experiments. B, effects of mono- and divalent cations on binding. The base-line condition of 8 mM Na^+ in 20 mM HEPES (pH 7.0) is indicated by a dashed line at 100%. Bar graphs depict the mean ± S.E. of three independent experiments.

are listed in table 1. All of the AAIs listed are full agonists, *i.e.*, they inhibit neuronally stimulated contractions in the MVD by greater than 90%. Whereas the potencies of AAIs in both the binding assay and the MVD preparation ranged over three orders of magnitude, the absolute potencies were generally higher in the functional assay. Such a difference is not unexpected for K_i binding values obtained from agonist-agonist binding assays, because only the affinities of antagonists competing for antagonist binding generally match biological potencies (Burt, 1985). A comparison of AAI binding affinities and their relative agonist potencies in the MVD preparation (fig. 6) reveals a significant correlation ($r = 0.97$; $P < .002$).

Effects of guanine and adenine nucleotides on AAI binding. [^{3}H]WIN 55212-2 (0.5 nM) binding in the presence of nonhydrolyzable analogs of GTP and ATP is shown in figure 7A. In the presence of 10 μM GTP-γ-*S*, specific binding was reduced 35 ± 1.6%, whereas 10 μM Gpp-NHp reduced binding by 30 ± 1.2%. Neither 10 μM ATP-γ-*S* nor App-NHp had an effect (0.13 ± 1.2 and 1.7 ± 3.8%, respectively). This selective modulation of ^{3}H-agonist binding is consistent with a receptor-G protein interaction (Abramowitz and Birnbaumer, 1982; Casey and Gilman, 1988).

Effect of monovalent and divalent cations. The effects of several ions on the specific binding of 0.5 nM [^{3}H]WIN 55212-2 in rat cerebellar homogenate is shown in figure 7B. The dashed line at 100% (fig. 7B) indicates specific binding observed by using the standard assay conditions of 20 mM HEPES-8 mM NaOH, pH 7.0. The decrease in specific binding caused by each of the monovalent cations was greatest at the highest concentration tested (100 mM). At this concentration, Na^+ decreased binding by 38 ± 1.9%; K^+ by 38 ± 2.3% and Li^+ by 33 ± 2.0%. Calcium caused a slight increase at low (1 mM) concentrations but none at 10 mM. Mg^{++} (1 mM) had the greatest effect on specific binding with a 2.5-fold increase; 100 mM Mg^{++} produced only a slight increase in binding compared to control. The degree of specific binding observed in a buffer containing (millimolar): Tris, 20, pH 7.3; NaCl, 120; KCl, 5; $CaCl_2$, 2; and $MgCl_2$, 1, showed no significant difference from that observed in 20 mM Tris, pH 7.3 (data not shown).

TABLE 2

Effect of various ligands (1 μM) on 0.5 nM [^{3}H]WIN 55212-2 binding

Each compound was assayed in triplicate.

Ligand	% Change in Binding	Ligand	% Change in Binding
Serotonergic		Muscarinic	
5-HT	−1	Atropine	−6
8-Hydroxy-2(di-*n*-propylamino)tetralin hydrobromide	+44	Oxotremorine	+1
Ketanserin	−1	Pirenzipine	−5
Trazodone	−5	Opioid	
Pindolol	−7	Morphine	−6
Dopaminergic		Naloxone	+9
Apomorphine	+11	±-Bremazocine	−5
Haloperidol	+13	D-Ala^2-D-leu^5-enkephalin	−3
Spiperone	+1	Eicosanoids	
GABA		Prostaglandin	
Baclofen	−2	D2	−2
±-Bicuculine	−10	E2	−0.1
Amino acids		F2	−0.1
Strychnine	−2	Leukotriene E4	+5
Taurine	−19	*Sigma*/PCP	
N-methyl-D-aspartate	+1	Rimcazole	−11
Kainic acid	+14	SKF-10047	−1
L-Glutamic acid	+8	−3-3-(3-hydroxyphenyl)-N-propylpiperidine	+2
Uptake sites		+3-3-(hydroxyphenyl)-N-propylpiperidine	+18
Desmethyl-imipramine	−8	1,3-di(2-tolyl)guanidine	+3
Imipramine	+0.3	Phencyclidine	+6
5-(*p*-nitrobenzyl)-6-Thioinosine	−1	+-MK-801	+3
Hormones		Neuroactive peptides	
Progesterone	+5	Bradykinin	−13
β-Estradiol	−6	Substance P	−10
Corticosterone	−5	Neurotensin	−1
D-Thyroxine	−3	Somatostatin	+4
Adenosine		Angiotensin II	−10
Adenosine	+1	Calcium channel	
2-Chloro-adenosine	−0.3	Diltiazem	−7
Benzodiazepine		Verapamil	+6
Diazepam	+1	Other	
RO15-1788	−5	Tiotidine	−6
Adrenergic		Thioinosine	+1
Prazosin	−2	Pyrilamine	−14
Clonidine	+10	Harmine	+1
Propranolol	+9	1-Methyl-3-isobutylxanthine	+0.2
		Forskolin	0

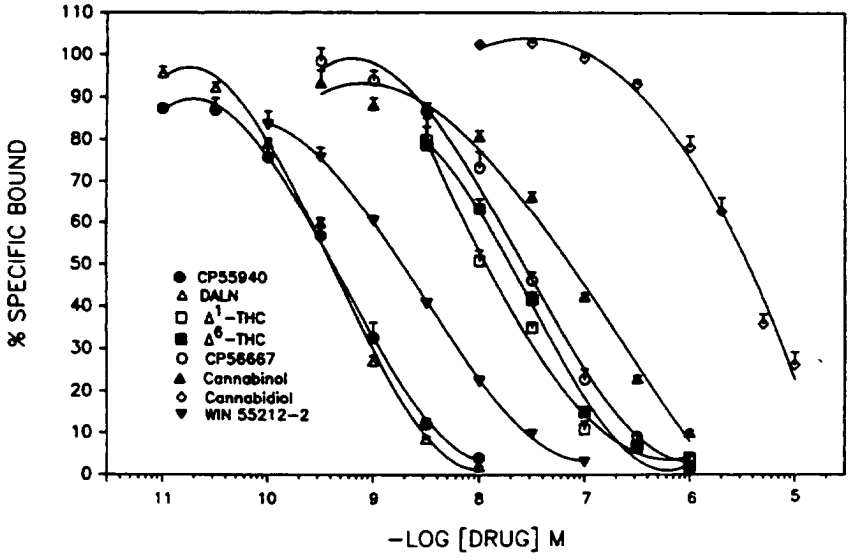

Fig. 8. Effect of various synthetic and natural cannabinoids on [^{3}H]WIN 55212-2 (0.5 nM) specific binding in rat cerebellar homogenate. ▼, Unlabeled WIN 55212-2 competition curve. All drugs reduced [^{3}H]WIN 55212-2 specific binding by greater than 90% except cannabidiol which could not be tested above 10 μM due to vehicle effects. Each data point is the mean ± S.E. of three experiments each performed in triplicate. DALN, desacetyllevonantradol.

TABLE 3

Cannabinoids: binding parameters from competition experiments of 0.5 nM [^{3}H]WIN 55212-2 in rat cerebellar membranes and IC_{50} values for inhibition of neuronally stimulated contractions in isolated MVD

Each value is the mean ± S.E. of at least three independent experiments.

Cannabinoid	Binding in Rat Cerebellum K_i	Slope Factor	MVD Assay IC_{50}
	nM		*nM*
DALN	0.26 ± 0.016	1.18 ± 0.023	2.3 ± 0.08
CP55940	0.35 ± 0.044	0.99 ± 0.042	0.3 ± 0.03
Δ^1-THC	10.2 ± 0.78	0.93 ± 0.089	4.0 ± 0.49
Δ^6-THC	16.5 ± 0.58	0.93 ± 0.070	9.4 ± 1.35[a]
CP56667	21.3 ± 2.40	0.99 ± 0.061	4.7 ± 0.4
Cannabinol	61.8 ± 1.08	0.84 ± 0.007	29 ± 2.7[b]
Cannabidiol	2,420 ± 323	1.42 ± 0.095	—[c]

[a] Maximal inhibition of twitch height = 69%.
[b] Maximal inhibition of twitch height = 74%.
[c] Maximal inhibition of twitch height was 50% at 1 μM.

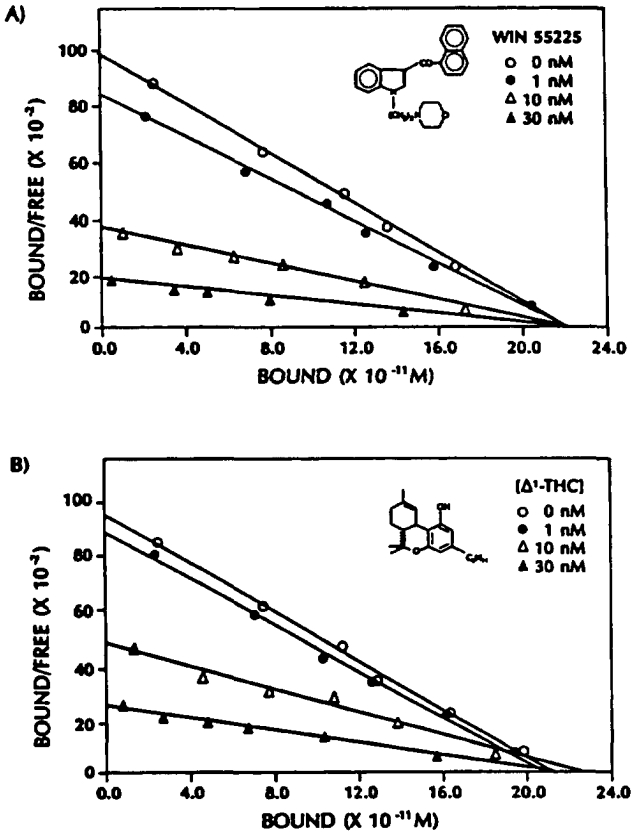

Fig. 9. Scatchard analysis of [^{3}H]WIN 55212-2 (0.25–22 nM) specific binding in the presence of various concentrations of WIN 55225 (A) or Δ^1-THC (B). Nonspecific binding was determined by using 1 μM WIN 55212-2. A representative pair of experiments from three independent studies is shown. Lines shown were determined by least-squares linear regression analysis.

Regional variation of binding density in the brain. Studies of the regional distribution of [^{3}H]WIN 55212-2 binding in the rat brain, by using a radioligand concentration of 20 nM, indicated the following densities (picomoles per milligram of protein, $n = 3$): cerebellum, 1.13 ± 0.060; hippocampus, 0.90 ± 0.112; striatum, 0.84 ± 0.051; cerebral cortex, 0.48 ± 0.007; hypothalamus, 0.40 ± 0.039; olfactory tubercle, 0.38 ± 0.031; thalamus, 0.31 ± 0.037; medulla/pons, 0.12 ± 0.009; and spinal cord, 0.076 ± 0.005.

Competition for AAI binding by putative neurotransmitters and other ligands. The specificity of [^{3}H]WIN 55212-2 binding in cerebellar homogenate is suggested by the inability of many common pharmacological agents to interfere, at a concentration of 1 μM, with the binding of [^{3}H]WIN 55212-2 (table 2). One exception was 8-hydroxy-2-(di-*n*-propylamino)tetralin hydrobromide, a 5-HT_{1a} agonist, which stimulated AAI binding by 44%; pinodolol, however, which also acts at 5-HT_{1a} and 5-HT_{1b}, sites had no effect on binding.

Effects of cannabinoids on AAI binding. In contrast to the other ligands tested, both natural and synthetic cannabinoids were able to compete for [^{3}H]WIN 55212-2 binding sites (fig. 8). The correlation coefficient of cannabinoid binding data and MVD inhibitory activity was 0.85 ($P < .05$) with the nonpsychoactive cannabidiol excluded (table 3). K_i values and slope factors (Hill coefficients) for the cannabinoids are listed in table 3.

Increasing concentrations of Δ^1-THC produced an effect on [^{3}H]WIN 55212-2 binding similar to that produced by increasing concentration of WIN 55225 (fig. 9). The apparent K_d increased from 1.8 to 6.9 nM in the presence of 30 nM Δ^1-THC, with no change in the value of the B_{max} (fig. 9B). Increasing concentrations of WIN 55225 produced apparent K_d values which increased from 1.8 nM in the absence of competing compound to 11.0 nM in the presence of 30 nM WIN 55225; the B_{max} values remained unchanged (fig. 9A).

Thus, radioligand saturation studies performed in the presence of increasing concentrations of an AAI, WIN 55225, or a cannabinoid, Δ^1-THC, produced similar patterns of competition (fig. 9, A and B). This pattern is typical of simple competitive inhibition as opposed to uncompetitive or noncompetitive inhibition; nevertheless, more complex models of ligand-receptor interactions cannot be ruled out by our current studies (Tomlinson, 1988).

Discussion

The binding site for the AAI [^{3}H]WIN 55212-2 meets the major criteria for a functional receptor in rat cerebellar membranes. The saturable binding indicates a finite number of sites which are differentially distributed in the central nervous system. Although the density of sites is high (1.2 pmol/mg of protein in cerebellum), this level does not exceed the densities seen for striatal dopamine (Boyson *et al.*, 1986), cortical benzodiazepine (Zezula *et al.*, 1988) or whole brain glutamate receptors (Greenamyre *et al.*, 1984). The concentration of cannabinoid receptors in a rat cortical P_2 preparation is reported to be 1.85 pmol/mg of protein (Devane *et al.*, 1988).

Binding of [^{3}H]WIN 55212-2 is specific (>90%), reversible and of high affinity (1.9 nM). Other compounds in this chemical series compete for the site with complete competition occurring over a concentration range of 2 to 3 log U. A similar number of binding sites is observed in the rat cerebellum for all of the AAIs tested.

As would be predicted for a protein, the binding site is sensitive to pH and to heat. Stereospecificity is shown by the fact that both (*R*)-WIN 55212-2 and the (*R*)-enantiomer of the 3-aryoyl paramethoxy analog (Analog 1A) of WIN 55212-2 bound with greater affinity than their (*S*)-enantiomers. This corresponds to the stereoselectivity observed in the MVD prep-

aration in which both (*S*)-enantiomers showed no agonist activity (Ward *et al.*, 1990b).

Binding characteristics such as high affinity, reversibility, saturability, stereospecificity, regional distribution and displacement with drugs of the same class have all been associated with binding to acceptor sites involved in uptake processes, active transport, enzymatic reactions or nonspecific binding (Laduron, 1984). The significant correlation between the affinities of AAIs for the [^{3}H]WIN 55212–2 binding site and their potencies as agonists for inhibiting neuronally stimulated contractions in the MVD suggests that the AAIs bind to a functional receptor.

The increased [^{3}H]WIN 55212–2 binding in the presence of divalent cations, particularly magnesium, is typical of the effect these ions have on the affinity of agonists at G-protein-linked receptors (Birnbaumer *et al.*, 1985). The monovalent cations, sodium, lithium and potassium decrease AAI binding. This pattern differs from that seen with opioid receptor subtypes (Pert and Snyder, 1974; Pfeiffer, 1982) and *alpha*-2 noradrenergic receptors (Greenberg *et al.*, 1978), both of which are coupled to inhibitory G-proteins (Birnbaumer *et al.*, 1985). In the case of the opioid and *alpha*-2 noradrenergic receptor assays, agonist binding affinities are decreased by sodium and lithium, but not by potassium. The nonselective effect of monovalent cations on AAI binding might not involve changes in receptor G-protein coupling because inhibition of adenylate cyclase by WIN 55225 is not dependent on sodium (Pacheco *et al.*, 1991).

The affinities of AAIs for the [^{3}H]WIN 55212–2 binding site (table 1) are also highly correlated with their potencies for inhibiting adenylyl cyclase in rat cerebellar membranes under both basal and forskolin-stimulated conditions (Pacheco *et al.*, 1991). This adenylyl cyclase inhibition is dependent on magnesium and GTP, attenuated by pertussis toxin and not dependent on nonhydrolyzable analogs of GTP. [^{3}H]WIN 55212–2 binding in rat cerebellar membranes was selectively inhibited by the nonhydrolyzable guanine nucleotides, GTP-γ-*S* and GppNHp and not by the corresponding adenine nucleotides, ATP-γ-*S* and AppNHp. The same stereoselectivity observed in the binding assay is apparent in the adenylyl cyclase assay in rat cerebellar membranes (Pacheco *et al.*, 1991).

Of the pharmacological agents tested, only natural and synthetic cannabinoids were found to compete for the [^{3}H]WIN 55212–2 binding site; this observation, in combination with the good correlation between AAI and cannabinoid binding affinities and their potencies in the MVD functional assay, suggests that AAIs may exert their antinociceptive properties, at least in part, through a mechanism related to the cannabinoids.

Because cannabinoids compete with [^{3}H]WIN 55212–2 for its binding site, it is interesting to consider various neurotransmitters which have been reported to interact with cannabinoids. Cannabinoid modulation of binding at the dopaminergic receptor, for instance, has been reported (Hillard and Bloom, 1982, 1984; Bloom and Hillard, 1985), but apomorphine, haloperidol and spiperone had no effect on [^{3}H]WIN 55212–2 binding. Likewise naloxone, [D-Ala^2,D-Leu^5]enkephalin, morphine and bremazocine had no effect on binding, although cannabinoids have been reported to interact with opioid receptors (Bloom and Hillard, 1985; Vaysse *et al.*, 1987).

It is highly likely that AAIs interact with the cannabinoid receptor described by Devane *et al.* (1988). This receptor, characterized with a synthetic, bicyclic cannabinoid possessing antinociceptive properties, [^{3}H]CP55940, shares several similarities with the AAI binding site. Both ligands appear to interact with a G-protein-coupled receptor. [^{3}H]CP55940 binding, like [^{3}H]WIN 55212–2 binding, is sensitive to nonhydrolyzable analogs of GTP which maintain a low-affinity state in receptors coupled to G-proteins (fig. 7; Devane *et al.* 1988). Both AAIs and classical cannabinoids inhibit adenylyl cyclase, cannabinoids in neuroblastoma cell membranes (Howlett, 1985) and AAIs in rat cerebellar membranes in which inhibition is GTP-dependent and sensitive to pertussis-toxin (Pacheco *et al.*, 1991).

The sequence obtained following the cloning of the gene for the cannabinoid receptor confirms that the receptor belongs to the class of G-protein-coupled receptors containing seven transmembrane domains (Matsuda *et al.*, 1990).

Both WIN 55212–2 and CP55940 reduce the amplitude of voltage-gated calcium currents in NG108–15 cells, a neuroblastoma-glioma cell line. Addition of CP55940 following maximal suppression by the WIN 55212–2 produced no further inhibition of calcium currents. Likewise, the WIN compound did not further inhibit calcium currents suppressed by preadministration of the CP compound (Mackie and Hille, 1992).

Regional mapping studies also support the suggestion that AAIs and cannabinoids bind to the same receptor. Autoradiographic studies of the regional distribution of [^{3}H]CP55940 receptors in the rat brain indicate high concentrations in cerebellum, cerebral cortex, hippocampus and basal ganglia with little binding in the brainstem (Herkenham *et al.*, 1990, 1991; Mailleux and Vanderhaeghen, 1992). Mapping of [^{3}H]WIN 55212–2 *in vitro* and by receptor autoradiography reveals a distribution very similar to that seen with the synthetic cannabinoid (Jansen, 1992). Furthermore, the synthetic cannabinoid DALN competitively inhibited the binding of [^{3}H]WIN 55212–2 (Jansen, 1992).

The binding affinities of several cannabinoids and AAIs for both the [^{3}H]CP55940 and [^{3}H]WIN 55212–2 binding sites are highly correlated ($r = 0.95$) (Howlett *et al.*, 1992; Kuster *et al.*, 1990; Ward *et al.*, 1990a). It has also been shown that the binding of [^{3}H]CP55940 is inhibited by WIN 55212–2 in a manner consistent with a competitive interaction: a concentration-dependent increase in K_d with no change in B_{max} (A. C. Howlett, personal communication); Δ^1-THC inhibits radiolabeled AAI binding in a comparable manner (fig. 9B).

Finally, cannabinoids and AAIs have similar effects *in vivo*. They share potential therapeutic applications including sedation and analgesia (Mechoulam, 1986; Ward *et al.*, 1988). Taken together, these data strongly suggest that the AAI binding site characterized in this report and the cannabinoid receptor described by Devane *et al.* (1988) are the same.

It is highly likely that inhibitory activity of the MVD is mediated *via* cannabinoid receptors, because the potency of cannabinoids in this assay is highly correlated with affinity for cannabinoid receptor binding sites labeled with [^{3}H]CP55940 ($r = 0.91$) (Ward *et al.*, 1990a). Potency of cannabinoids in the MVD assay broadly parallels psychoactive potency in humans. The same is true for other *in vitro* measures of cannabinoid receptor affinity or activity such as [^{3}H]CP55940 binding (Devane *et al.*, 1988) and inhibition of adenylyl cyclase (Howlett, 1985). Thus, psychoactive molecules such as Δ^1-THC and Δ^6-THC are inhibitory in the MVD preparation; the more potent psychoactive molecule levonantradol is also more potent than THC in the MVD preparation; the mildly psychoactive mole-

cule cannabinol is weaker in the MVD preparation and has partial agonist profile, and the nonpsychoactive molecule cannabidiol produces less than 50% inhibition in the MVD.

The observation that the AAI binding site shares several characteristics with the cannabinoid receptor suggests a common site of action which could account for the observed antinociceptive properties of this series of compounds. The chemical structure of AAIs, an indole nucleus with a aroyl group substituted at the 3-position and an aminoalkyl group on the nitrogen (Bell *et al.*, 1991), has no easily discerned similarity to the structures of classical cannabinoids. The affinities of AAIs for the [^{3}H]WIN 55212-2 binding site indicate a pharmacophore distinct from that suggested by Howlett *et al.* (1988) for cannabinoids (Estep *et al.*, 1990).

It is suggested that AAIs may provide a novel class of compounds for studying the cannabinoid receptor including, perhaps, a unique approach to the development of cannabinoid antagonists and the elucidation of cannabinoid receptor subtypes.

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